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14. ABSTRACT

The specific aims of this project are to: 1**)** determine the efficacy, pharmacokinetics, toxicology and imaging capacity of RGD-targeted Nexil; and, 2) determine the ability of other ovarian cancer-specific targeting ligands to enhance the efficacy of Nexil. Substantial progress has been made on both of these specific aims. This progress report covers work done during the first 10 months of the grant. Significant achievements to date include: the design and synthesis of two different linkers to be used in the coupling with the RGD peptide to the Nexil polymer; the synthesis of cyclic RGD peptide using Fmoc strategy on solid support; the coupling of cyclic RGD to linkers, deprotection and documentation of purity; synthesis of a set of molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule; establishment of a fluorescence polarization assay for assessment of affinity of binding to soluble integrins; addition of a fluorochrome to RGD-linker-polymer to facilitate pharmacokinetic and tissue distribution studies; and, establishment of a strategy for the synthesis of Lyp-1 using a solid support strategy.

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Efficacy, pharmacokinetics, toxicology, imaging capacity

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Table of Contents

Page

Introduction…….……..….. 4

Body…….. 4

Key Research Accomplishments……..…….. 11

Reportable Outcomes…… 11

Conclusion…… 11

References……. 12

Title: Targeting Paclitaxel-Loaded Nanoparticles to Ovarian Cancer
Grant #: W81XWH-09-1-0223
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INTRODUCTION

The specific aims of this project are to: 1**)** determine the efficacy, pharmacokinetics, toxicology and imaging capacity of RGD-targeted Nexil; and, 2) determine the ability of other ovarian cancer-specific targeting ligands to enhance the efficacy of Nexil. Substantial progress has been made on both of these specific aims. This progress report covers work done during the first 10 months of the grant.

This report covers the period from the effective date of this research contract (July 1, 2009) through March 16, 2010.

The success of Abraxane in increasing the delivery of placitaxel (PTX) to breast cancers has sparked interest in other nanoparticle-based delivery systems that might out-perform Abraxane. CT-2103 (Xyotaq) consists of a polyglumatic acid (pGA) polymer to which PTX has been loaded to 37 %(w/w). This drug has proven safe, but has not yet been shown to have improved efficacy in phase 3 trials. We have further improved on CT-2103 by modifying the pGA backbone such that each glutamic acid in the polyer has an additional glutamic acid linked to as a side chain (pGGA). This polymer can also be loaded to a high level with PTX (35%); however, the key advantage is that the pGGA polymer loaded with PTX (pGGA-PTX) spontaneously forms a 20 nm particle in aqueous solutions and plasma. This molecule, now known as Nexil^{TM}, increased plasma exposure by 24-fold over that attainable with an equimolar dose of free PTX, and in the H460 lung cancer model it increased delivery of PTX ($AUC_{0-\infty}$) by 68-fold (1). We have now shown that Nexil significantly outperforms both free paclitaxel and Abraxane with respect to efficacy in multiple tumor (2).

The rationale for attaching tumor-targeting ligands to Nexil derives from the very large increase in affinity attained when multiple ligands work together to produce a Velcro-like effect. While the association rate constant increases linearly with the number of ligands, the dissociation rate falls exponentially such that the overall affinity increases markedly. The affinity can become so high that, once the dendrimer is bound, it cannot be displaced even by very high concentrations of the free ligand.

KEY RESEARCH ACCOMPLISHMENTS

Despite of the improvements in the cancer treatment concerning surgical intervention, radiation and chemotherapeutic drugs, development of efficient delivery of therapeutic systems is lagged behind. This subject has been actively reviewed and it is of common agreement that nanotechnology represents an excellent opportunity to move forwards the drug delivery research (3-6).

Among the systems currently being investigated, the polymeric nanoparticles conjugates have already demonstrated promising application (4). These macromolecular prodrugs comprise a minimun of three components: a natural or synthetic water-soluble polymeric carrier, a biodegradable polymer-drug linkage and bioactive antitumor agent. In this sense, a polyglutamate polymer loaded with paclitaxel (CT-2103, Xyotax) was described in 1998 with good *in vivo* antitumor activity (7). However, although favorable phase II clinical trial results,

three randomized phase III trials in patients with non-small cell lung cancer failed to demonstrate an improvement in either progression-free or overall survival and CT-2103 has not yet received marketing approval.

Based on a polyglutamic acid polymer backbone, we have developed a new polymer where a glutamate side chain has been added to each monomer in the polymer to create polyglutamylglutamate (PGGA). When PTX is conjugated to this polymer to an extent of 35% (w/w) to create poly-(_-L-glutamylglutamine)–paclitaxel (PGGA–PTX), the tendency of the hydrophobic PTX molecules to interact with each other causes the polymer to collapse to form a nanoparticle of »20 nm in aqueous solutions as determined by dynamic light scattering (2) Figure 1.

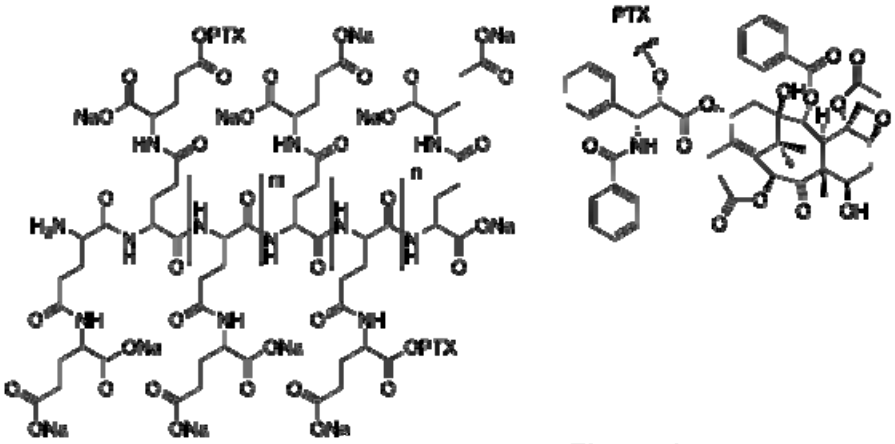


Figure 1

In our studies, this new nanoparticle have exhibited better efficacy compared to Abraxane (clinically approved paclitaxel formulation) and better pharmacokinetics parameters compared to native paclitaxel (2, Wang, 2010 #14307). It is very likely that the mechanism, which this nanoparticle acts over cancerous cells, is through a passive way. Passive targeting exploits the characteristic features of tumour biology that allow nanocarriers to accumulate in the tumor by the enhanced permeability and retention (EPR) effect. However, it is very desirable that drug delivery systems (DDS) could also actively target cancerous cells. In principle, this active approach could be performed by conjugating DDS with molecules that bind to receptors specifically over-expressed on the target cells.

The **overall goal** of this research project is to enhance the therapeutic efficacy of Nexil by targeting it to either the activated endothelial cells in tumors for anti-angiogenesis therapy, or to tumor cells themselves using peptides. The peptide chemical space is enormous and peptides are versatile in structure and conformation and highly pure peptides can be synthesized in large quantities compared to the protein ligands. Peptide ligands also generally display higher affinities for target receptors than the small molecule ligands. Although the Nexil nanoparticles are already extensively and rapidly endocytosed by tumor cells, our **hypothesis** is that selectively can be substantially further improved using the capability of the pGGA polymer to carry a large number of tumor-specific peptides per molecule. Our present goal is to combine this novel polyglutamylglutamate nanoparticle containing paclitaxel with the known cyclic peptide (RGD). This peptide presents good binding affinity to $\alpha_v ß_3$ integrin receptors, which is overexpressed in tumor cells. Thus, it is expected that this new system would be able to deliver its payload (paclitaxel) to tumor cells in a very selective manner.

Specific Aim #1: RGD targeting

Integrins are involved in a large number of fundamental cellular processes such as cell-matrix adhesion, differentiation, proliferation, apoptosis. An important feature of these proteins, which has attracted the attention of the scientific community,(8-9) is the fact that certain classes of integrins are over-expressed on tumor cells and on the endothelial cells of their capillaries. Thus, these receptors could be used as specific targets to deliver bioactive compounds against tumors in a selective way.

Based on the natural ligand of the $\alpha_v ß_3$ integrin (vitronectin), Kessler et al, (10) have developed a cyclic peptide containing the important recognition-binding motif (arginine-glycine-aspartic acid, RGD) present on the vitronectin structure. Molecules presenting this RGD amino acid sequence and RGD mimetic can inhibit many integrins and have been considerably studied. Using the cyclic RGD peptide as tumor targeting moiety, we envisioned the possibility of synthesizing a novel conjugate based on the polyglutamylglutamate (PGGA) backbone containing paclitaxel as bioactive compound (Figure 2).

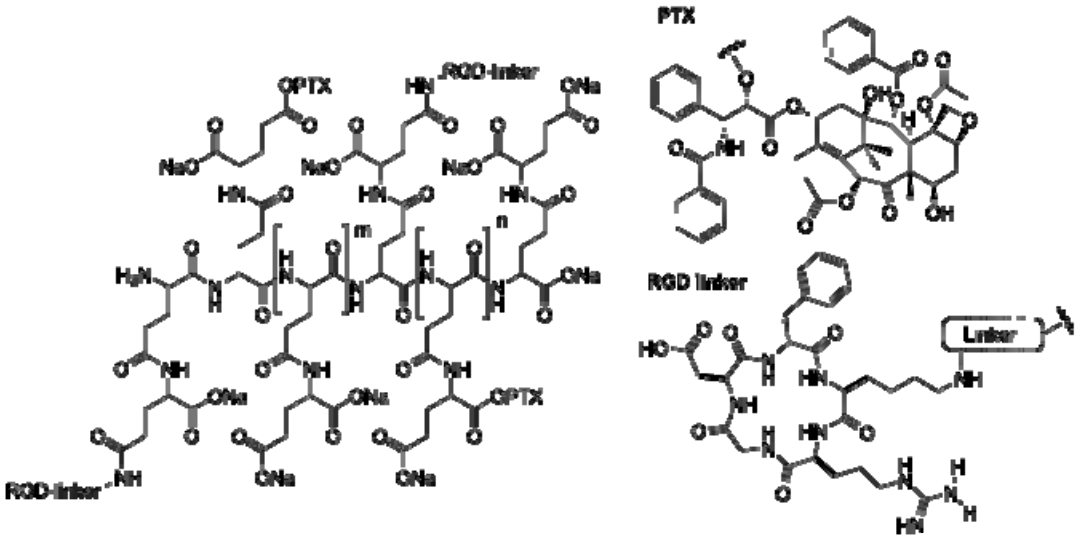
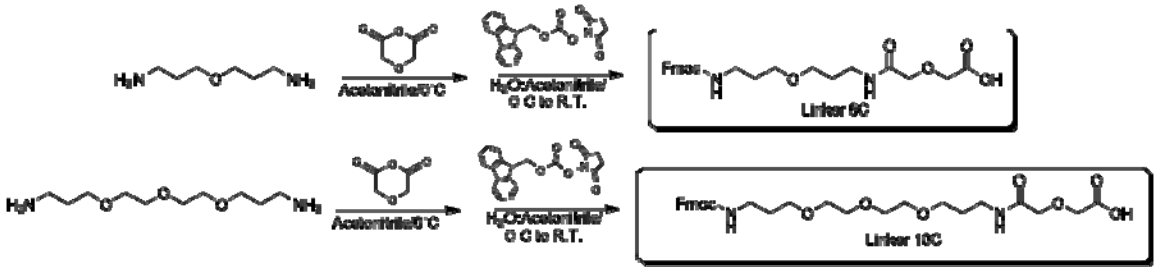


Figure 2

An important aspect to take into account in the proposed structure depicted on Figure 2 is the linker length. Precedents in the literature suggest that RGD binding activity to integrin decreases with longer linkers.(11)

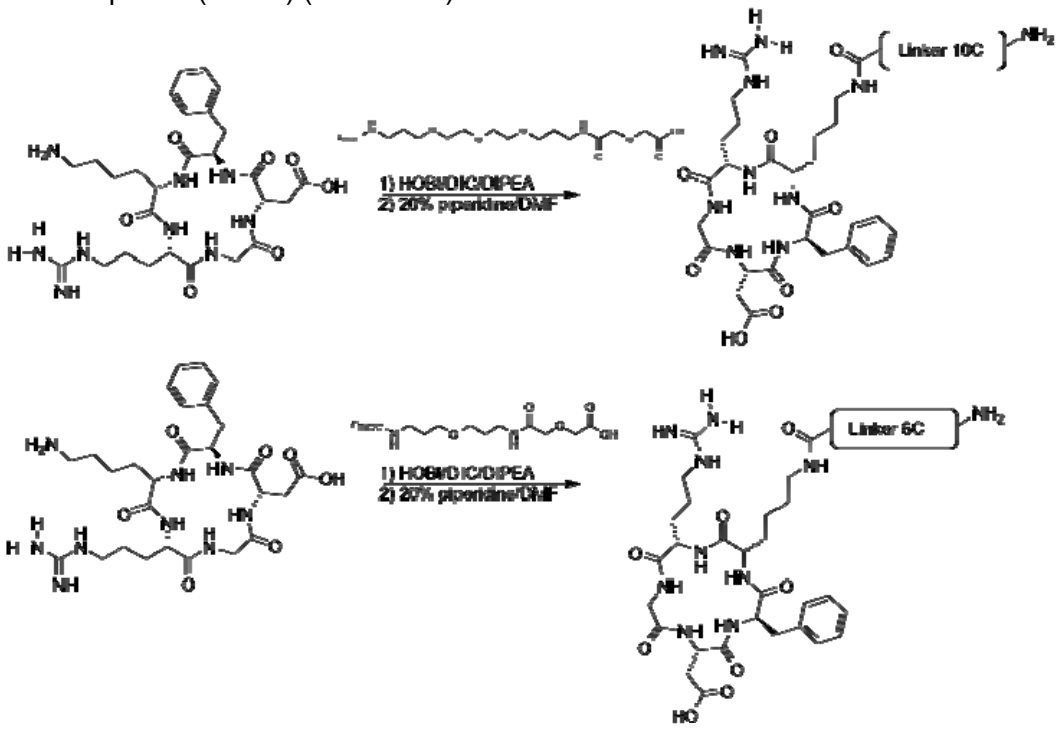
We have designed and synthesized two different linkers to be used in the coupling with the RGD peptide (Scheme 1).



Scheme 1

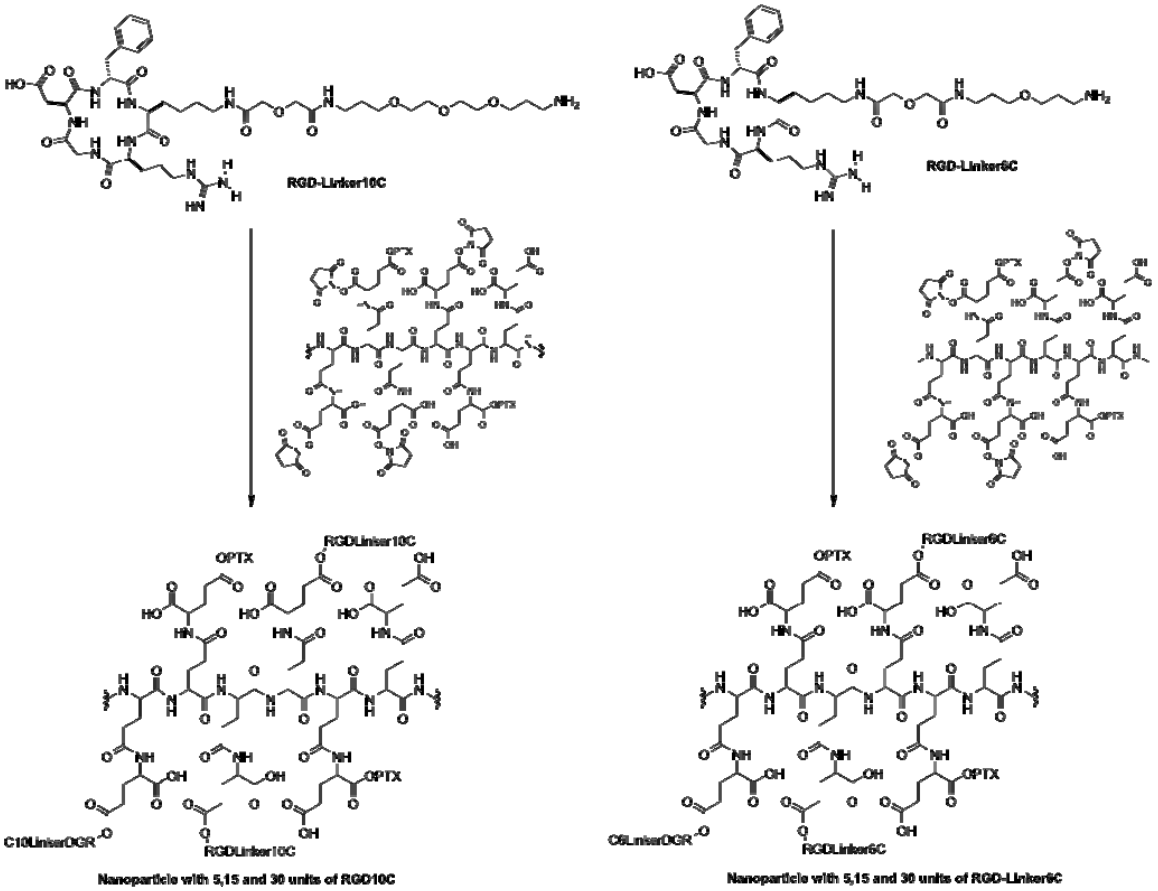
Following the procedure described by Kessler et al,(10) the synthesis of the cyclic RGD peptide was done using Fmoc strategy on solid support (2-chlorotrityl chloride resin) and the cyclization step was performed in solution phase. The ^{1}H-NMR and the mass spectra of the peptide obtained are in agreement with the literature.

With both linkers (Linker 10C and Linker 6C) and the cyclic RGD peptide in our hands, we have proceeded to the coupling reaction between the carboxylic acid functionality present on the linkers and the lysine amino acid residue on the peptide. Using standard peptide coupling conditions and after purification in the HPLC (reverse phase), we obtained the RGD peptide coupled with the linkers in the protected form (Fmoc group). The deprotection of the Fmoc group was done using a 20% piperidine solution in DMF and the final product purified by reverse phase (HPLC) (Scheme 2).



Scheme 2

In parallel, the carboxylate groups present on the polyglutamylglutamate (PGGA) backbone were activated with N-hydroxysuccinimide (NHS) for the reaction with RGD-Linker. Based on in a previous experiment with a different system (unpublished results), the optimal loading amount of RGD peptide is 15 units per carrier molecule. In this sense, we have synthesized a set of molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule (Scheme 3). These molecules will be assayed *in vitro* with purified $\alpha_v ß_3$ integrin to determine their binding activity using a fluorescence polarization experiment (FPA) (12).



Scheme 3

On the other hand, fluorescent versions of the molecules depicted on Scheme 3 are also being synthesized. To this aim, a part from the RGD-Linker molecules coupled to polyglutamylglutamate backbone, the known carboxyfluorescein was also attached to the polymer structure. A set of fluorescent molecules containing the RGD-Linker10C has already been synthesized and the set with RGD-Linker6C is currently undergoing.

These fluorescent molecules will be used in a cell-based experiment with HUVEC cells(13) to determine ability of the conjugates to target tumor cells overexpressing $\alpha_v ß_3$ integrin receptors. The FPA experiment and the cell-based assay will provide valuable information about the ability of these new conjugates to target selectively tumor cells.

Depending on the bioactivity of these conjugates, the best molecules will be synthesized in gram scale for being assayed *in vivo* experiments. It will be analyzed not only the capability of these new conjugates to target tumor, but also the efficiency of these systems to deliver their paclitaxel payload.

Specific Aim #2: Lyp-1 targeting

Lyp-1 is a member of a novel class of peptides that can markedly and selectively increase the penetration of drugs into tumor nodules (14). Lyp-1 was originally isolated from a phage display library on the basis of its ability to direct phage to MDA-MB-435 tumor xenografts. (15) This compound has proved to bind specifically to tumor lymphatic and tumor cells, leading

to cell death by apoptosis and inhibiting tumor growth in mice bearing breast cancer xenografts. The attractive feature of Lyp-1 is the ability of this compound to be internalized by tumor cells, which can be explored for drug delivery purposes.(16) Recently, Ruoslahti et al have suggested the internalization mechanism of Lyp-1 is through the known receptor p32 that is present and overexpressed at the tumor cell surface (17).

Our goal in this Specific Aim is to synthesize Lyp-1 and use it to enhance targeting of Nexil to tumors (Figure 1).

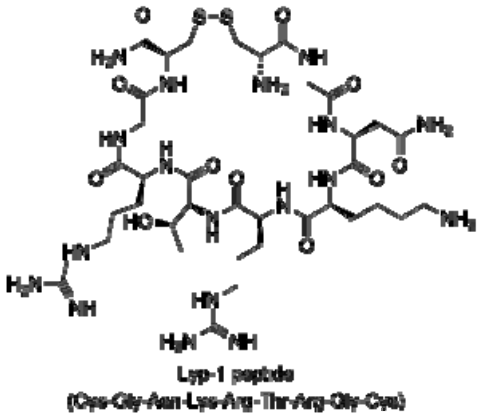


Figure 1

Precedents in the literature support our hypothesis that the efficacy of Nexil can be further improved using Lyp-1 as targeting moiety. Nanoparticles of iron oxide,(18) albumin-based (19) and even baculovirus combined with Lyp-1 have demonstrated excellent results for binding tumor cells (20).

We are currently developing a synthetic strategy where the total synthesis of Lyp-1 coupled to two different linkers can be performed on solid support. This approach, apart from the intrinsic benefits of the synthesis on solid support, would favor the intramolecular disulfide bond in the cyclization step. Scheme 1 depicts our first approach to Lyp-1 synthesis.

Scheme 1

The crude peptide was analyzed by LC/MS and MALDI/TOF techniques; however only by MALDI/TOF was it possible to observe the expected mass of the product (992, M^++1). The crude product was purified using the preparative HPLC system (reverse phase column). Unfortunately after the purification the product isolated did not show the mass of the desired compound, moreover the chemical yield obtained was poor (less than 1%). Analyzing the synthetic route proposed, it is very likely that although very mild conditions (1% of TFA in DCM) were used the acidic treatment in the cyclization step was strong enough to cleave the peptide from the solid support leading to the low yield obtained in the final step.

The solid support chosen in this first approach was the 2-chlorotrityl-chloride resin. This is a well-known resin suitable for the attachment of cysteine as first amino acid, minimizing the racemization tendency of this amino acid. However the acidic sensitivity of this resin can be a pitfall in this synthetic route even at very mild conditions. To solve this problem the same synthetic sequence will be used in a different solid support, Rink amide or Wang resin, which are more resistant to acidic conditions.

KEY RESEARCH ACCOMPLISHMENTS

- Further validated Nexil as a platform for drug delivery.
- Designed and synthesized two different linkers to be used in the coupling with the RGD peptide (PROPRIETARY).
- Synthesized cyclic RGD peptide using Fmoc strategy on solid support.
- Coupled cyclic RGD to linkers, deprotected and documented purity.
- Synthesized a set of molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule.
- Established fluorescence polarization assay for assessment of affinity of binding to soluble integrins.
- Added fluorochrome to RGD-linker-polymer to facilitate pharmacokinetic and tissue distribution studies.
- Established a strategy for the synthesis of Lyp-1 using a solid support strategy.

REPORTABLE OUTCOMES

Presentations

None

Abstracts

None

Manuscripts

None

CONCLUSIONS

We have established that RGD can be successfully loaded onto linkers that appear to be good candidates for attaching RGD in variable amounts to the Nexil backbone, and have prepared lots containing 5, 10 or 30 units per polyglutamylglutamate polymer. These are now ready for testing to determine whether the variation in RGD loading significantly alters binding to soluble and/or cell surface integrins. The next step in this project will entail in vivo studies of the relative extent to which addition of RGD enhances the delivery of paclitaxel to the H-460 lung cancer xenograft model. The Lyp-1 peptide is proving somewhat of a challenge to synthesize; however, we have identified several next steps that are likely to overcome this challenge that are actively being pursued at the present time.

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